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The N-terminal half of the heavy chain of botulinum type A neurotoxin forms channels in planar phospholipid bilayers

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The heavy chain of botulinum type A neurotoxin forms channels in planar phospholipid bilayer membranes. Channel activity is confined to the N-terminal half of this chain; the C-terminal half is inactive. Channel activity is stimulated by low pH (4.5–5.5) on the *cis* side (the side to which protein is added), neutral pH on the opposite *(trans)* side, and *cis* positive voltages. These findings are strikingly similar to those previously reported for analogous fragments of diphtheria and tetanus toxins.

Neurotoxin fragment; pH-dependent channel; Voltage gating; Lipid bilayer; Diphtheria toxin; Tetanus toxin; *(Clostridium botulinum)*

1. INTRODUCTION

The botulinum neurotoxins, which number among the most potent toxins known, cause a flaccid paralysis by blocking the release of acetylcholine from presynaptic cholinergic nerve terminals. The toxin is synthesized by the anaerobe *Clostridium botulinum* as a single polypeptide (M_r ~150000) which is split (termed nicking) into a heavy chain (M_r ~100000) and a light chain (M_r ~50000) by a protease endogenous to the bacteria or by mild trypsinization; the two chains are separated by reduction of the disulfide bond(s) which link(s) them (fig.1). The seven serologically distinct botulinum neurotoxin types recognized so far have a similar structure (see [1] and [2] for a general review of botulinum toxin). Interestingly, tetanus and diphtheria toxin share the same general macrostructure with botulinum neurotoxin [1].

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Although a clear picture of the mechanism by which the toxin gains entry to the cytosol has yet to emerge, there may be an analogy with diphtheria toxin which is believed to employ cell-surface receptor binding, receptor-mediated endocytosis, and membrane translocation of its enzymatic light chain into the cytosol from an acidic vesicle compartment [3,4]. The exact molecular mechanism by which transmitter release is disabled also remains a mystery, although type D botulinum toxin has recently been shown to ADP-ribosylate a membrane protein of M_r ~21000 in bovine adrenal gland homogenate, suggesting that the mechanism is enzymatic [5].

It has previously been demonstrated that the heavy chains of botulinum type B neurotoxin,

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tetanus toxin and diphtheria toxin form large, voltage-dependent and pH-dependent ionic channels in planar lipid bilayers [6]. The channel-forming properties of the three toxins are remarkably alike, with channel activity maximal under the pH conditions which are likely to exist in an endocytic vesicle. This comparison makes compelling the suggestion that these pores formed by the heavy chain are involved in protein translocation of the light chain, and a possible role as 'tunnel proteins' has been suggested [6].

In this study we report that the heavy chain of botulinum type A neurotoxin, like that of type B, makes voltage-dependent and pH-dependent ionic channels in planar lipid bilayers. We further show that channel-forming activity is confined to the N-terminal half of the heavy chain; the C-terminal half of the heavy chain is devoid of channel-forming activity.

2. MATERIALS AND METHODS

2.1. *Neurotoxin and neurotoxin fragments preparation*

Botulinum type A neurotoxin was produced and purified as described [7], and its heavy and light chains were separated and purified chromatographically [8]. To cut the heavy chain, the whole neurotoxin (M_r ~145000) was digested with trypsin (EC 3.4.4.4) at a 10:1, w/w, ratio in 0.02 M Na_2HPO_4-NaH_2PO_4 buffer, pH 6.0, for 90 min at 30°C. The primary cleavage products were (i) light chain (M_r ~53000) linked to the N-terminal half of the heavy chain (M_r ~50000) by a disulfide bond (hence total M_r ~103000) and (ii) the C-terminal half (M_r ~47000) of the heavy chain; in fig.1 the two halves of the heavy chain are marked as H_2 and H_1. The two fragments (M_r ~103000 and ~47000) were purified by ion-exchange chromatography. The light chain (L) was then separated from the N-terminal half of the heavy chain (H_2), and the two were purified by ion-exchange chromatography. The two halves of the heavy chain were partially sequenced for characterization [9]. Details of fragmentation, purification and amino acid sequence determination will be published elsewhere (Sathyamoorthy, DasGupta, Niece and Foley, in preparation). The first 27 amino acid residues of the H_2 fragment (M_r ~50000) of the heavy chain [9] were identical to the N-terminal sequence of the intact heavy chain (M_r ~97000) [10]. This proved that (i) this fragment is the N-terminal half of the heavy chain, and (ii) the other half (M_r ~47000), whose 12 amino acid residues were sequenced [9], is apparently the C-terminal half of the heavy chain (H_1 fragment).

2.2. *Membrane formation and measurements*

Planar phospholipid bilayer membranes were formed at room temperature from the union of two lipid monolayers across a hole (0.1 to 0.2 mm diameter) in a Teflon partition [11] that had been pretreated with squalene; the partition separated two 1 ml compartments of a Teflon chamber containing buffered salt solutions, which were stirred independently by magnetic fleas. Monolayers were spread from 1% lipid solutions in hexane, and the solvent was allowed to evaporate before membrane formation. The lipid solutions consisted of either diphytanoylphosphatidylcholine (DPhPC) or a mixture of plant phosphatidylethanolamine (PE), plant phosphatidylcholine (PC), and bovine phosphatidylserine (PS) in the ratio PE/PC/PS of 2:2:1; all lipids were obtained from Avanti Polar Lipids, Birmingham, AL. The salt solutions contained 1 M KCl, 5 mM $CaCl_2$ and 0.1 mM EDTA. The *cis* solution (the one to which the toxin fragment was added) was buffered either at pH 4.7 with 5 mM dimethylglutaric acid (DMG) or at pH 5.5 with 5 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes). The *trans* solution was buffered either at pH 7.4 with 5 mM Hepes or with the same buffer as in the *cis* solution. In the latter case, the pH of the *trans* solution was sometimes raised during the course of an experiment by stirring into it small aliquots of concentrated Hepes solution. After membrane formation, neurotoxin fragments were added from stock aqueous solutions to the *cis* compartment, to final concentrations of 0.1–1 μg/ml.

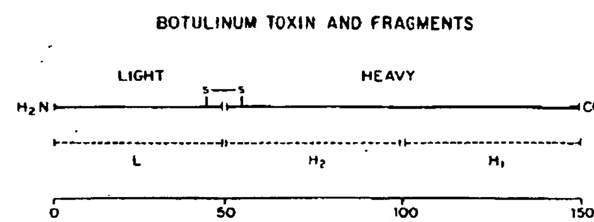

Fig.1. Botulinum neurotoxin and fragments. The scale is in units of kDa.

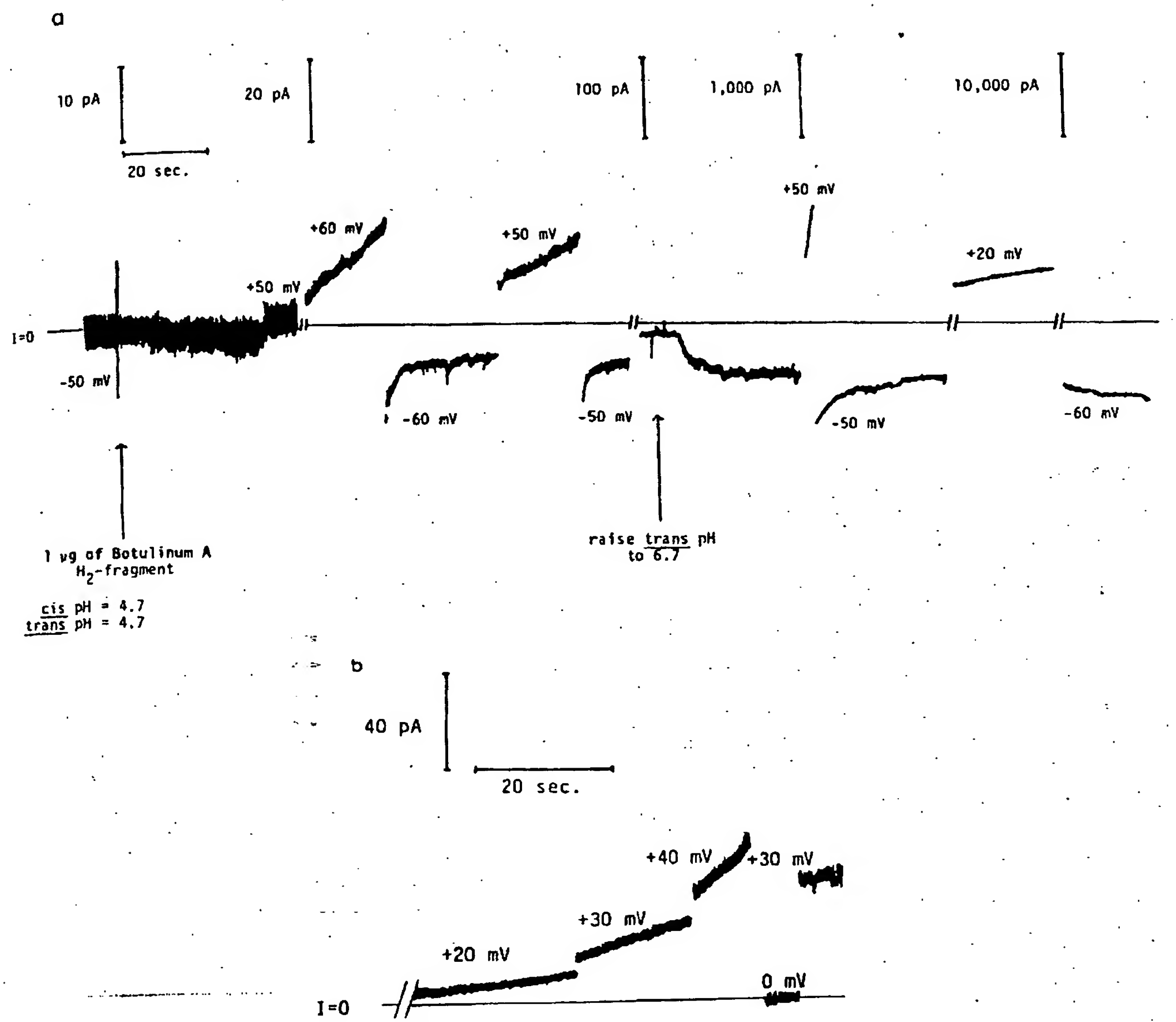

Fig.2. (a) Effect of raising *trans* pH on rates of channel opening and closing. Current traces are shown at different applied voltages. In the absence of toxin fragment, membrane current (and conductance) is virtually zero. Upon addition of ~1 μg of H_2 fragment to the *cis* compartment (volume 0.8 ml), the rate of channel opening (or closing) is seen as an increase (or decrease) in current with time. Rates of channel opening and closing at symmetric low pH (4.7) are compared to rates after raising the *trans* pH to 6.7. The rapid (5–10 s) increase in current (in the negative direction) seen immediately after raising the *trans* pH (see second arrow) is probably due to an increase in single-channel conductance. Further, the rate of channel opening at + 50 mV is increased by more than 50-fold over that before the elevation of the *trans* pH (note change in current scale). Solutions: 1 M KCl, 5 mM $CaCl_2$, 0.1 mM EDTA, in 5 mM DMG buffer, pH 4.7. *trans* pH was raised by addition of Hepes buffer. Lipid: PE/PC/PS (2:2:1 ratio). Voltage: All voltages refer to those of the *cis* side with respect to the *trans* side, which is defined as zero. (b) Effect of positive voltage on rate of channel opening. 10 min prior to the start of the record, H_2 fragment was added to the *cis* compartment to a concentration of ~0.2 μg/ml. Solutions: *cis*, 100 mM KCl, 5 mM Mes, pH 5.5; *trans*, 100 mM KCl, 5 mM Hepes, pH 7.4. Lipid: DPhPC.

Stock solutions of fragments were stored at 4°C at concentrations of ~150 μg/ml. Protein concentrations were estimations based on absorbance at 278 nm or intensity of Coomassie blue stained bands in polyacrylamide gels. Electrical measurements were made under voltage-clamp conditions using a single pair of Ag/AgCl electrodes, contacting the solutions via 3 M KCl agar bridges; current was monitored on a Narco physiograph chart recorder. The conductance (G) at any time is obtained from the relation $G = I/V$, where V is the voltage at which the membrane is clamped and I is the resulting current. The *trans* compartment was held at virtual ground; all voltages, therefore, refer to those of the *cis* compartment.

3. RESULTS

Several fragments of botulinum type A neurotoxin were examined for possible channel-forming activity: (i) the entire heavy chain; (ii) the N-terminal half of the heavy chain (H_2 fragment); (iii) the N-terminal half of the heavy chain linked via a disulfide bond to the light chain (H_2-L fragment); and (iv) the C-terminal half of the heavy chain (H_1 fragment) (see fig.1). The first three of these fragments listed were fairly similar in their channel-forming activity; differences which were evident include noise/fluctuation levels and potencies. In particular, experimental records with the heavy chain and the H_2-L fragment exhibited much more noise than those of the H_2 fragment. In addition, these fragments had to be present in ~5–10-fold higher concentrations than the H_2 fragment to achieve comparable conductances. When the C-terminal half of the heavy chain was examined under conditions which yielded maximal activity for the other fragments, no channel-forming activity was observed even when present at ~50-fold higher concentrations.

Addition of the H_2 fragment to one side of a planar lipid bilayer separating salt solutions at symmetric low pH (*cis*, 4.7; *trans*, 4.7) and subsequent clamping of the membrane potential to positive voltages results in steady rates of channel turn-on. (A typical record is shown in fig.2.) If the membrane is initially held at large negative potentials (< −50 mV), little or no activity is seen. Raising the *trans* pH causes a dramatic (>100-fold) increase in the rate of channel turn-on. This effect is apparent when one compares the rate of current increase at +50 mV at symmetric low pH to the rate after the *trans* pH has been raised to 6.7 (fig.2a, note the change in current scales). A second effect of raising the *trans* pH is a rapid (within stirring time) 3–5-fold increase in the steady-state conductance (fig.2a, second arrow). This is most likely due to an increase in the conductance of the single channels which comprise the macroscopic conductance, rather than an increase in the actual number of open channels. Consistent with this is a comparable rapid fall in conductance seen upon lowering of the *trans* pH from ~7.0 to ~5.0, presumably the result of a decrease in the single-channel size. No activity is exhibited by any of the active fragments when added to one side of a membrane separating solutions at symmetric neutral pH (or higher). If the *cis* pH is subsequently lowered to ≤5.5, however, full activity appears.

The effects of negative potentials on the channel kinetics are somewhat complicated (fig.2a). At symmetric low pH, large negative voltages (< −50 mV) result in a fast (2–5 s) phase of turn-off, followed by a much slower turn-off phase. This effect is also seen after the *trans* pH has been raised, but only initially; after ~1 min, potentials of −60 mV result in a turning-on of channels. It is likely that multiple open and closed states exist and that raising the *trans* pH drives channels into deeper open states. In addition to the gating of these channels by pH, there is also an effect of voltage on the rate of channel turn-on, with increasing positive voltages resulting in higher rates of turn-on (fig.2a,b).

4. DISCUSSION

It has previously been shown that botulinum neurotoxin types A, B, C_1, D and E [12,13], diphtheria toxin [14], and tetanus toxin [6,15] all form pH-dependent and voltage-dependent channels in planar lipid bilayer membranes. Furthermore, this channel-forming activity was found to be confined to the heavy chains of diphtheria toxin, tetanus toxin and botulinum type B neurotoxin [6,15,16]. In the present study, we have extended this finding to the heavy chain of botulinum type A neurotoxin. Moreover, we have shown that the

channel-forming domain of the heavy chain is restricted to its N-terminal half (the H_2 fragment); the C-terminal half does not possess channel-forming activity. (Although our sample of the light chain was too dilute to adequately test for channel-forming activity, it is known from previous work [6] that the light chain of botulinum type B neurotoxin does not form channels.) This is precisely what was found earlier for diphtheria toxin and tetanus toxin [6,15], thereby strengthening the analogy among these three toxins.

Several striking similarities among the channel-forming properties of the botulinum, tetanus and diphtheria toxins are worthy of mention: (i) comparable fragments (the N-terminal half of the heavy chain) form channels; (ii) the channels manifest a similar voltage dependence, particularly the increase in channel activity with *cis* positive voltages; (iii) there is a requirement of low *cis* pH in channel formation; (iv) channel activity increases upon elevation of the *trans* pH; and (v) the single-channel conductance increases with the elevation of *trans* pH. With regard to this last point, although we have not yet investigated botulinum type A neurotoxin at the single-channel level, effects of pH on macroscopic records (see fig.2a) are consistent with those seen previously with botulinum type B neurotoxin (and with diphtheria toxin and tetanus toxin as well) [6,13].

A question which remains unanswered is the possible connection between channel formation by the heavy chain and protein translocation of the light chain. In addressing the general question of protein translocation one seeks to find the means by which nature solves the problem of overcoming the significant energy barrier which polar, hydrophilic regions must overcome in their journey across the low dielectric medium of the plasma (or vesicle) membrane. In the light of this, and the similarity of conditions required for channel formation in bilayers and intoxication in cells, it has been suggested that the aqueous pores formed by the heavy chains of these toxins may accommodate the passage of the light chains in an unfolded conformation [6,16].

The genes for both tetanus toxin [17] and diphtheria toxin [18,19] have been cloned and sequenced and the primary structures of these toxin proteins thereby deduced. In recent years, the use of molecular cloning techniques in the study of ionic channels has provided a unique probe into the molecular mechanisms which underlie channel-forming activity. We hope to eventually bring this technique to bear on the channels formed by the botulinum neurotoxins.

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